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IMMUNE DIFFUSION ANALYSIS OF THE EXTRACELLULAR SOLUBLE ANTIGENS OF TWO STRAINS OF *RHIZOBIUM MELILOTI*

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ABSTRACT

DUDMAN, W. F. (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia). Immune diffusion analysis of the extracellular soluble antigens of two strains of *Rhizobium meliloti*. J. Bacteriol. **88**:782-794. 1964.—Immune diffusion techniques applied to cultures of two strains of *Rhizobium meliloti* grown in liquid defined medium showed the presence of multiple antigens. Improved resolution of precipitin patterns was obtained with concentrated antigens separated from the cultures as the extracellular soluble fraction or as suspensions of washed cells. The extracellular fraction contained the same diffusible antigens as the washed cells, but additional antigens were found in the cells after ultrasonic disruption. The extracellular soluble antigens were shown by analysis to contain polysaccharide and protein components. In immune diffusion systems, they gave rise to three groups of precipitin bands, two of which were characterized as polysaccharides by their susceptibility to periodate oxidation, and the third as protein by its lability to heat. All the extracellular antigens of both strains were shared except a fast-diffusing polysaccharide, which was specific for each strain. Despite the sharing of all but one of their antigens, cells of these strains showed only a low degree of cross-agglutination, suggesting that their surfaces are dominated by the specific polysaccharide. No differences could be found in the composition of the polysaccharides in the unfractionated extracellular antigens of the two strains, the main components of which were glucose (66%) and galactose (12%) in the presence of several other unidentified sugars in smaller amounts. The pattern of precipitin bands produced in immune diffusion systems by the extracellular soluble fraction could be changed by altering the cultural conditions.

The difficulty of recognizing *Rhizobium* strains is a major problem in studies of symbiosis, especially under field conditions, where it is necessary to distinguish known strains from those occurring naturally in the soil. Serological methods have been widely used, and many agglutination studies have been reported in the literature (e.g., Bushnell and Sarles, 1939; Vincent, 1941, 1942; Hughes and Vincent, 1942; Kleczkowski and Thornton, 1944; Purchase, Vincent, and Ward, 1951; Koontz and Faber, 1961; Vintikova, Šrogl, and Škrdleta, 1961; Johnson and Means, 1963). A major weakness in identification by agglutination techniques is the frequency with which cross-reactions occur, making positive identification of strains difficult.

Gel immune diffusion techniques, capable of giving more detailed information of antigenic structure than the classical serological methods, may be of value in the identification of *Rhizobium* strains. In this paper, the application of such techniques to the analysis of the extracellular soluble antigens of two strains of *R. meliloti* is described, and information about their chemical nature is presented. Because workers elsewhere are known to have experienced difficulty in applying immune diffusion techniques to the study of *Rhizobium*, some aspects of technique and some preliminary observations with the present system are given in detail to suggest how these difficulties may be overcome.

MATERIALS AND METHODS

Organisms. R. meliloti strains B and F were used (Dudman, 1964).

Media. For injection into rabbits, cultures were grown on the defined solid medium of Bergersen (1961) for 5 days at 25 C. For the preparation of antigens for analysis, the organisms were grown in the defined liquid medium described by Dudman (1964) containing 1% mannitol, 1% sodium glutamate, 1% sodium succinate, mineral salts, thiamine, and biotin.

Preparation of extracellular soluble antigens. Cul-

tures were grown in the defined liquid medium in 100-ml volumes in 250-ml indented conical flasks at 25 C on a rotary shaker operated at 150 to 200 rev/min. The cultures were harvested after about 8 days, after polysaccharide production had been determined to be at the maximal level by use of the anthrone method (Dudman, 1964). The viscous cultures were centrifuged on a Spinco model L ultracentrifuge at 78,000 × *g* for 30 min, and the clear supernatant liquids were decanted and reduced by about one-half in a rotary evaporator in which the temperature did not rise above 30 C. The solutions were dialyzed against repeated changes of distilled water at 4 C, and then were centrifuged again as above. The solutions were finally dried from the frozen state to yield the extracellular soluble antigens.

Preparation of polysaccharide. Cultures grown in the liquid medium were centrifuged and dialyzed as described above. Three volumes of ethanol were added to the solutions to yield gelatinous precipitates of polysaccharide plus protein. The precipitates, isolated from 2 liters of culture, were dissolved in 300 ml of water and buffered by the addition of 5 ml of acetic acid and 10 g of sodium acetate. The solutions were then deproteinized in the Sevag (1934) manner by repeated shaking with 50 ml of chloroform plus 10 ml of *n*-butanol. After five cycles of shaking, the aqueous solutions were centrifuged at 78,000 × *g* for 1 hr. The supernatant fluids were dialyzed, and the antigens were isolated by drying from the frozen state.

Hydrolysis of polysaccharides and chromatographic analysis. Samples (10 to 20 mg) of the antigens were hydrolyzed with 2 ml of 1 N sulfuric acid in sealed ampoules immersed in boiling water for 5 to 7 hr, neutralized with barium carbonate, and centrifuged; the clear supernatant solutions were applied to Whatman no. 1 paper. To ensure detection of minor sugar components, the hydrolyzed samples were applied in amounts equivalent to 0.5 mg of antigen. The papers were irrigated with *n*-butanol–benzene–pyridine–water (5:1:3:3, v/v) solvent by descending chromatography. The papers, after drying, were sprayed with the silver nitrate reagent of Trevelyan, Procter, and Harrison (1950) or with aqueous aniline oxalate (Horrocks and Manning, 1949).

Antigens for immunization. Saline suspensions of cultures grown on agar were disrupted by treatment with a Mullard 20-kc/sec ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London, England). Electron microscopy (Fig. 1) showed that 20-min treatment was sufficient to break more than 99% of the cells in 3 ml of culture suspension containing 1 mg/ml of cell dry matter.

Antisera. Rabbits were immunized with a primary intramuscular injection, in the hindquarters, of 1 ml of an emulsion containing equal parts of Freund's complete adjuvant (Difco) and the disrupted culture suspensions; 1 month later, the rabbits were given a secondary injection in the ear vein of 1 ml of the saline suspension without adjuvant. The animals were bled from the ear on the third, fifth, seventh, and tenth days after the second injection. The antisera were sep-

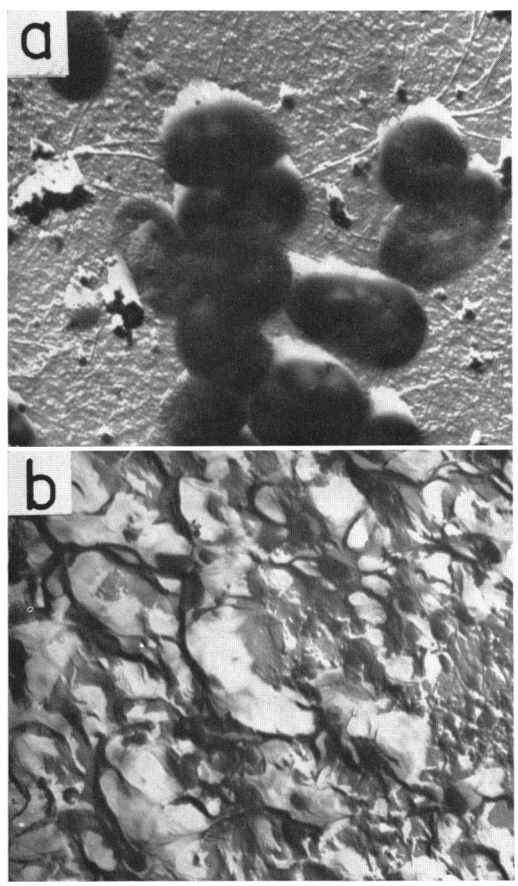


FIG. 1. *Ultrasonic disruption of Rhizobium meliloti. Electron micrographs showing cells of strain B (a) before and (b) after treatment (× 19,000). Very similar results were obtained with strain F.*

arated from the clots and stored, without addition of any preservative, at −20 C.

Variations in precipitin patterns have been shown to arise from the use of different antisera prepared against the same antigen (Carlisle, Hinchliffe, and Saslaw, 1962). To avoid such variations, all the experiments reported in this paper were carried out with one antiserum, against each strain of *Rhizobium*, obtained from a single bleed of a single rabbit. Both antisera used here were obtained from the fourth bleeds of the respective rabbits.

Absorbed antisera. Absorbed antisera were prepared by adding 1 ml of dense suspension of *Rhizobium* cells of the appropriate strain, washed four times with cold saline, to 1 ml of the desired antiserum. The mixtures were incubated at 37 C for 1 hr and kept overnight at 2 C, after which they were centrifuged to yield absorbed antisera.

Immune diffusion. The gels were prepared with Oxoid Ionagar No. 2 at 0.75% final concentration in a variety of buffers. However, except where otherwise stated, all the diffusions shown in the figures were carried out in gels containing 0.85% sodium chloride. Sodium azide (0.025%) was incorporated in the gels as a preservative. The use of 1% sodium azide, sometimes recommended in gels for diffusion, was detrimental to the formation of precipitin bands. Because many of the antigens in the present study were polysaccharides, better precipitin patterns were obtained with larger quantities of reactants than are usually found necessary for protein antigens. This was achieved by using gel layers 4 mm thick and wells 4 mm in diameter. Smaller wells could be used for the concentrated antigen solutions but not for the antisera. Initially, the diffusions were carried out in a refrigerator, but the development of bands was later found to be equally good and more rapid at 25 C.

The patterns of bands were recorded photographically by use of the immune diffusion viewing box described by Crowle (1961) as the source of illumination.

Immune electrophoresis. The electrophoresis was performed on the microscale (Scheidegger, 1955) in gels (2 mm thick, 8 cm long) made with Oxoid Ionagar No. 2 (0.75%) in barbital acetate or borate buffer, both made to ionic strength 0.05 and pH 8.6. An electrophoresis tank of the type described by Fazekas de St. Groth, Webster, and Datyner (1963) was used. The subsequent immune diffusion was carried out at 25 C.

RESULTS

General observations on immune diffusion with Rhizobium antigens. Because of the multiplicity of buffer systems described in the literature for immune diffusion gels (e.g., Crowle, 1961), a comparison was made of the precipitin bands produced by one *Rhizobium* immune system in gels made with the following nine electrolyte solutions: 0.85% sodium chloride, isotonic phosphate (pH 7.3, μ = 0.15), isotonic tris(hydroxymethyl)-aminomethane(tris; pH 7.3, μ = 0.15), isotonic barbital (pH 7.5, μ = 0.15), barbital acetate (pH 8.6, μ = 0.05), barbital acetate (pH 8.6, μ = 0.10) plus 1% sodium azide, borate (pH 8.6, μ = 0.05), and tris-ethylenediaminetetraacetate-borate (pH 8.8; Aronsson and Grönwall, 1957). All the gels were made with 0.75% agar, and the immune system used was a 0.5% solution of the extracellular soluble antigens of *R. meliloti* strain B and anti-B antiserum. Two types of double diffusion were compared, that in plates (Ouchterlony, 1958) and in tubes (Preer, 1956). The tube double diffusion in saline agar was set up in duplicate at 25 and 4 C to observe the effect of temperature.

From this experiment, the following general conclusions were drawn. (i) The *Rhizobium* immune system was sensitive to temperature. The development of precipitin bands in tubes was complete in 3 to 4 days at 25 C but required 8 days at 4 C. This scale of time difference was later confirmed also for double diffusion in plates. However, the same patterns of bands were obtained at both temperatures. (ii) All but two of the electrolyte systems used here were satisfactory and gave similar diffusion patterns of similar density. The Aronsson and Grönwall (1957) buffer allowed the appearance of all the precipitin bands, but all were of reduced density. The addition of 1% sodium azide to barbital acetate buffer inhibited the formation of most of the precipitin bands. (iii) Precipitin bands developed more slowly in the tubes than in the plates. Because of this, and the inconvenience inherent in the tube technique, plate diffusions were used in all subsequent immune diffusions.

Examination of cultures and culture fractions. Cultures of the two strains of *R. meliloti*, fully grown on solid or liquid media, were unsatisfactory for use directly in immune diffusion plates. The patterns of precipitin bands were indistinct, making comparison of the strains uncertain. The

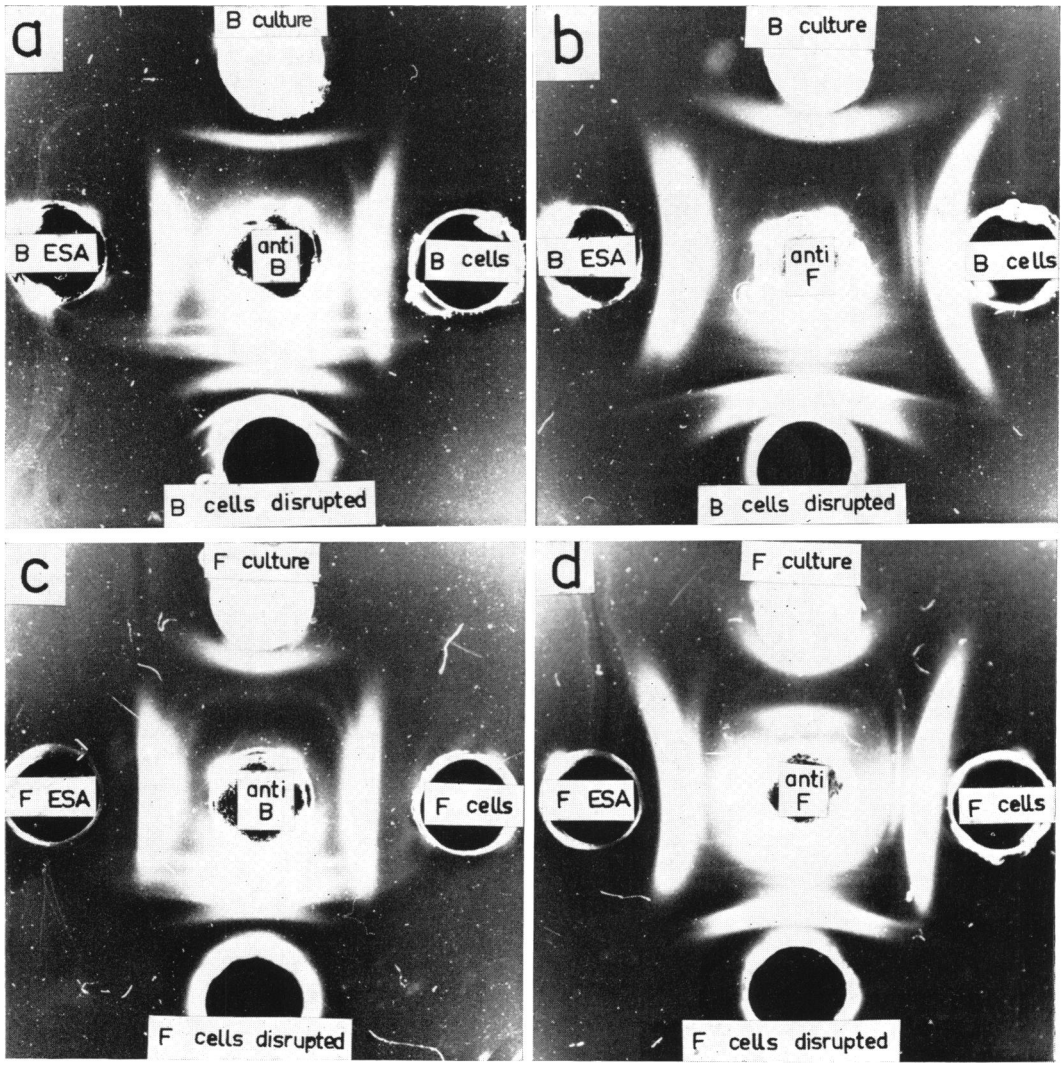
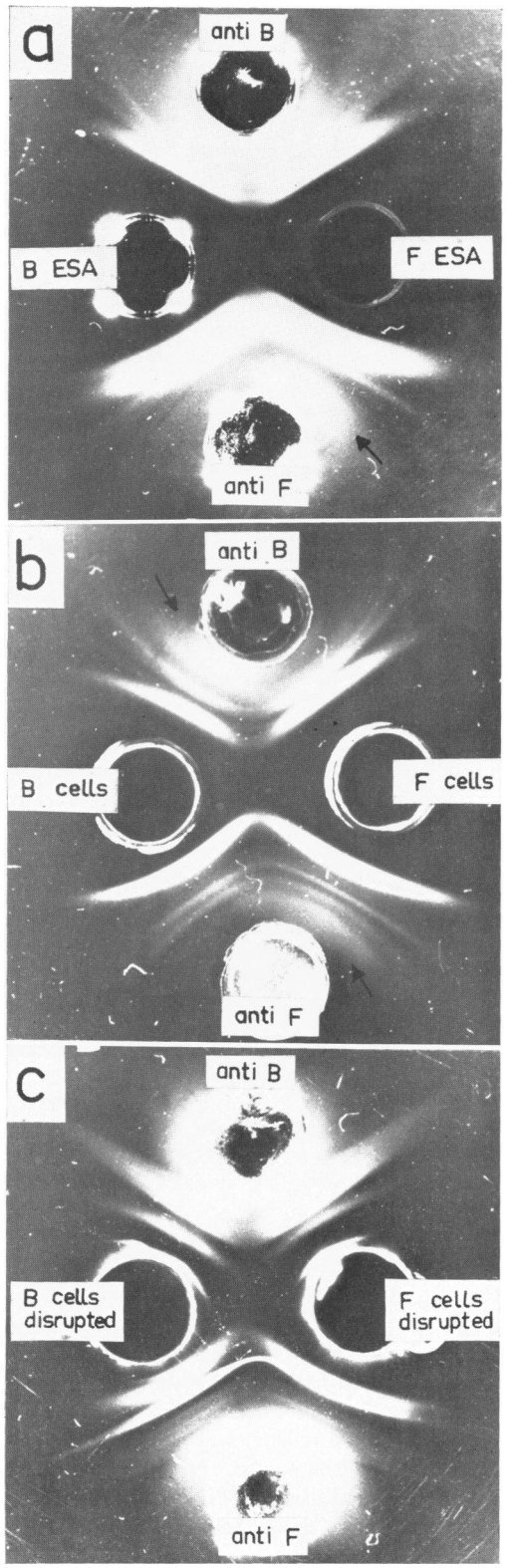


FIG. 2. *Immune diffusion analysis of Rhizobium meliloti strain B and strain F. (a) B antigens and anti-B antiserum, (b) B antigens and anti-F antiserum, (c) F antigens and anti-B antiserum, (d) F antigens and anti-F antiserum. Antigens: cultures grown 9 days in defined liquid medium, washed cell suspensions (180 mg/ml of cells, dry weight), ultrasonically disrupted cell suspensions (180 mg/ml), and extracellular soluble antigens (ESA; 25 mg/ml). Photographed after 4 days at 25 C.*

relatively low concentration of antigens in the cultures was probably responsible; the influence of antigen concentration on the immune diffusion patterns of these *Rhizobium* systems is shown below.

Much clearer patterns of precipitin bands were obtained in immune diffusions with concentrated antigens consisting of suspensions of washed cells, suspensions of ultrasonically disrupted cells, and with the extracellular soluble antigens. Figure 2 shows the results obtained with these antigens of the two strains when diffused against their homologous and heterologous antisera. The bands produced by the extracellular soluble antigens were similar to those given by the washed-cell suspensions, but additional bands were revealed by the disrupted cells. It will be seen that strain B and strain F share most of their antigens. This is shown more directly in Fig. 3, where the antigenic fractions of both strains are compared si-



multaneously with homologous and heterologous antisera. The patterns of bands from the same antigenic fractions of the two strains were identical except for a specific antigen in each, which was seen as a diffuse band close to the homologous antiserum well. These specific bands are more clearly seen in photographs to be presented below.

Despite the sharing of so many antigens, the two strains exhibited a low degree of cross-reaction when examined by agglutination techniques (Evans, 1957). In qualitative macroscopic slide tests with antisera diluted 1:5, washed cells of the two strains rapidly agglutinated with their homologous antisera but did not show any tendency to agglutinate with the heterologous antisera. Cross-agglutination to a low titer was detected, however, in a tube agglutination test. Cells of strain B and strain F cross-agglutinated with the heterologous antisera to titers of 1:40, but agglutinated with their respective homologous antisera to titers of 1:1,600 and 1:3,200, respectively.

Examination of the extracellular soluble antigens. Because of the poor results obtained in immune diffusions with the cultures and the more informative precipitin patterns obtained with the concentrated culture fractions, these fractions were investigated more carefully. Attention was given first to the extracellular soluble antigens, i.e., all the antigens remaining in the supernatant liquid after cultures grown in the liquid defined medium were centrifuged to remove the particulate antigens (cells and flagella). Care was taken during the isolation procedure to minimize the risk of inactivating any labile antigens that may have been present.

The marked influence of antigen concentration on the precipitin pattern in immune diffusion against the homologous antisera is shown in Fig. 4. With the antigens dissolved at the maximal concentration of 25 mg/ml in physiological saline, at least five bands were detected in the extracellular soluble antigens of strain B and at least six bands in those of strain F. The number of bands decreased as the concentration of the

FIG. 3. *Comparison of antigens of Rhizobium meliloti strain B and strain F in immune diffusion with anti-B and anti-F antisera. (a) Extracellular soluble antigens (ESA; 25 mg/ml), (b) washed-cell suspensions (180 mg/ml of cells, dry weight), and (c) ultrasonically disrupted cells (180 mg/ml). Photographed after 42 hr at 25 C. Bands arising from the strain-specific antigens are indicated, where visible, by arrows.*

antigenic mixture applied to the gel decreased. At concentrations of 0.4 mg/ml, or less, only a single band was detected in either strain. No attempt was made to enumerate the bands or antigens, because it is believed that some of the bands contain more than one component.

The complexity of these antigenic mixtures was further revealed when they were examined by immune electrophoresis (Fig. 5). Each antigen appeared to be electrophoretically heterogeneous. The inflections in the precipitin bands suggested

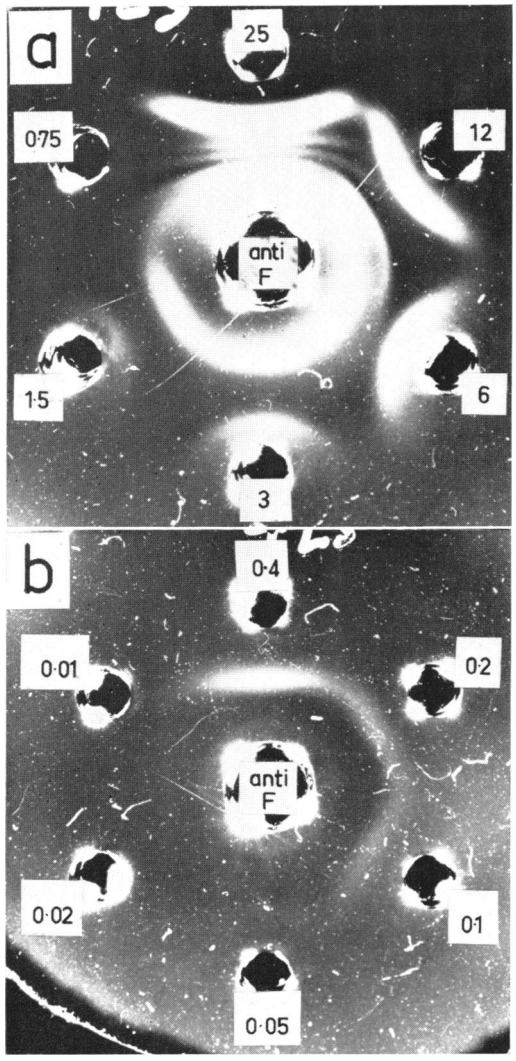


FIG. 4. *Influence of antigen concentration (in mg/ml) on the precipitin pattern obtained in immune diffusion with the extracellular soluble antigens of strain F. Photographed after 4 days at 25 C. Very similar results were obtained with strain B.*

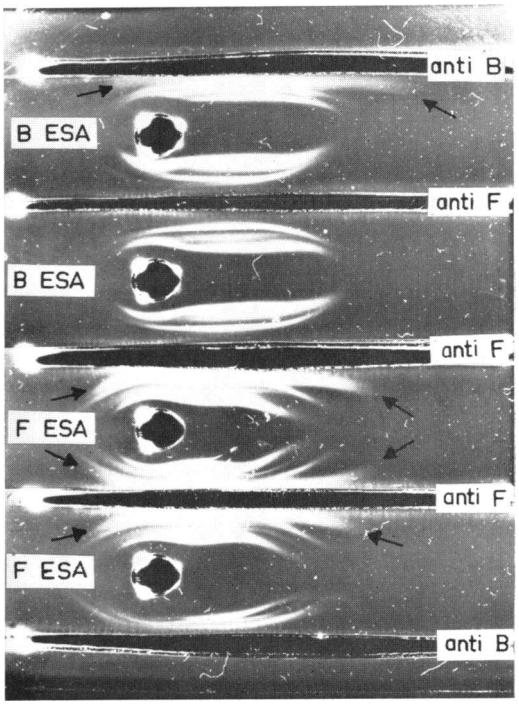


FIG. 5. *Immune electrophoresis of the extracellular soluble antigens (ESA) of strain B and strain F. Electrophoresis (anode to the right) for 2 hr at 1.5 v/cm, 48 ma, in 0.75% agar in barbital acetate buffer (pH 8.5, ionic strength 0.05). Photographed after 24-hr immune diffusion at 25 C. The strain-specific antigens are indicated by arrows.*

that most of the antigens were present in forms that give rise to two electrophoretic mobilities, one of which remained at the origin and another that migrated towards the anode. In addition, the fraying of the ends of the major bands indicates further, lesser heterogeneities. However, none of the antigenic components migrated sufficiently to separate from other components. Very similar electrophoretic patterns were obtained with the extracellular soluble antigens of the two strains when reacted against the homologous antisera, but, when the antigens were diffused against the heterologous antisera, the bands nearest the serum troughs were missing in the case of both strains. The significance of these strain-specific antigens is discussed below.

Nature of the antigens. Chemical analysis of the unfractionated extracellular soluble antigens of the two strains of *R. meliloti* showed that they consisted mainly of polysaccharide with a small amount of protein (Table 1).

TABLE 1. *Analysis of freeze-dried preparations of the extracellular soluble antigens (ESA) of R. melilioti strain B and strain F*

Determination	Strain B		Strain F	
	ESA	Sevag-[a] treated ESA	ESA	Sevag-[a] treated ESA
	%	%	%	%
Total N	0.78	0.17	0.69	0.15
Protein (N × 6.25)	4.9	1.1	4.3	0.9
Polysaccharide[b]	53	64	72	79
Reducing sugar[c]	63	74	70	80
Glucose[d]	59	66	59	66
Galactose[d]	11	12	8	12

[a] Deproteinized by the method of Sevag (1934).

[b] Anthrone determination, calculated as "glucose."

[c] Determined after 7-hr hydrolysis with 1 N sulfuric acid, calculated as "glucose."

[d] By elution from paper chromatograms and anthrone determinations.

Determination of total carbohydrate by the anthrone method (Fairbairn, 1953) on the unhydrolyzed materials gave values of 53 and 72%, calculated as glucose, for the antigens of strains B and F, respectively. This was supported by hydrolysis of samples with 1 N sulfuric acid at 96 C until maximal reducing values (Somogyi, 1952; Nelson, 1944) were obtained, when values equivalent to 63 and 70% glucose were found for the two strains, respectively.

Because no hexosamine was found in these antigens, and there was no evidence for the presence of nucleic acid components in the ultraviolet-absorption spectra, it was assumed that the nitrogen content found by a micro-Kjeldahl method indicated the presence of 4 to 5% protein. This was supported by the detection of about 15 amino acids in hydrolysates by paper chromatography and spraying with a ninhydrin reagent (Moffat and Lytle, 1959). Furthermore, treatment of solutions of the antigens for the removal of protein by the Sevag (1934) method reduced the nitrogen content of these anitgens to the equivalent of about 1% protein. After this deproteinization treatment, the anthrone and reducing sugar values increased significantly.

When examined for component sugars, the hydrolysates of both strains were very similar in qualitative and quantitative composition. Glucose and galactose were the main constituents of the antigens from both strains. A number of minor components with faster R_F values than glucose were detected, together with a possible trace (about 0.5%) of a uronic acid. Of particular interest was a fast-moving component (R_F, 0.81) with the chromatographic mobility of a tetramethylated sugar; in the solvent used, the mobility of tetramethyl glucose was R_F, 0.84.

Nature of the antigens in the precipitin bands. When the extracellular soluble antigens were found to contain polysaccharide and protein constituents, an attempt was made to determine whether the antigens in the various precipitin bands in the immune diffusion plates could be identified as either polysaccharide or protein.

Lability to heat was used as an indication of protein antigens. Solutions containing 25 mg/ml of the respective antigens in saline were heated at 96 C in sealed bottles for 1 hr. Immune diffusion showed that the intermediate precipitin bands had been destroyed by this treatment (Fig. 6). The fast- and slow-diffusing antigens, nearest the antiserum and antigen wells, respectively, were heat-stable, but the density of the fast-diffusing component appeared to have been increased relative to that of the same band in the unheated control samples, whereas the density of the slow-diffusing band was less.

The effect of heat on the bands and their densities was investigated further. Solutions containing 25 mg/ml of the antigens, dissolved in isotonic phosphate buffer (pH 7.3), were heated at 96 C, and samples were removed after 30, 60, and 120 min for analysis by immune diffusion. Figure 7 shows the results obtained with the antigens of strain F; very similar results were obtained with strain B. The heat-labile antigens were destroyed within the first 30 min, but the fast-diffusing antigens, nearest the antiserum well, were not destroyed even after 2 hr of boiling. The heat treatment made these antigens diffuse at a faster rate; the precipitin bands formed by these antigens in the heated samples were well developed within 17 hr after setting up the diffusion, whereas the corresponding bands in the untreated sample had not yet formed.

The slow-diffusing antigens, nearest the antigen well, were degraded by the heat treatment into two components. One remained at the position of the original band, and its density decreased progressively with the duration of heating; the

second component appeared as a new band that diffused more rapidly as the length of heat treatment increased.

The heat-stable antigens were susceptible to oxidation by periodate when treated under conditions based on those described by Tinelli and Staub (1959). Samples (100 mg) of the freeze-dried extracellular soluble antigens of the two strains were dissolved in 10 ml of water, and 10 ml of 1 M meta-periodic acid were added. The stoppered flasks were kept in a refrigerator at 2 C, and samples were taken daily for determination of residual periodate by the method of

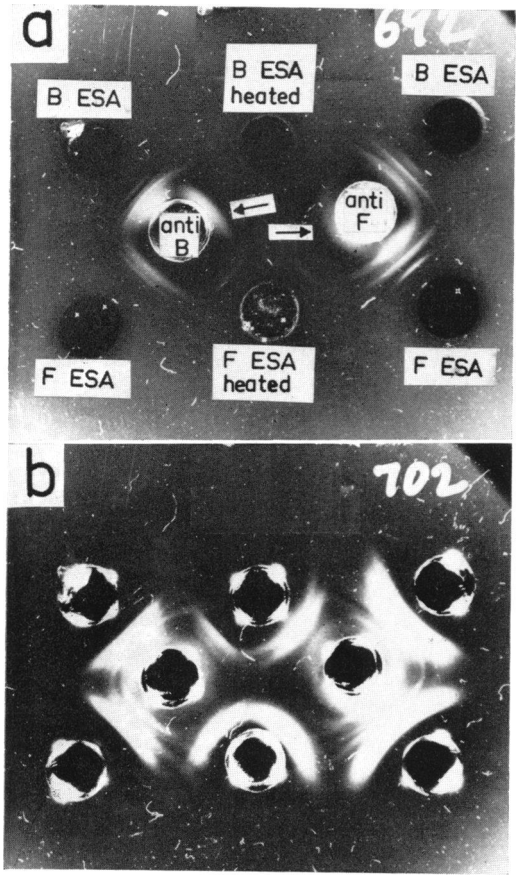


FIG. 6. *Effect of heat (1 hr at 96 C) on solutions (25 mg/ml) in 0.85% sodium chloride of the extracellular soluble antigens (ESA) of strain B and strain F. Agar (0.75%) in barbital acetate buffer (pH 8.5, ionic strength 0.05). Photographed after (a) 16 hr and (b) 5 days at 25 C. Note the heat-stable strain-specific antigens in (a).*

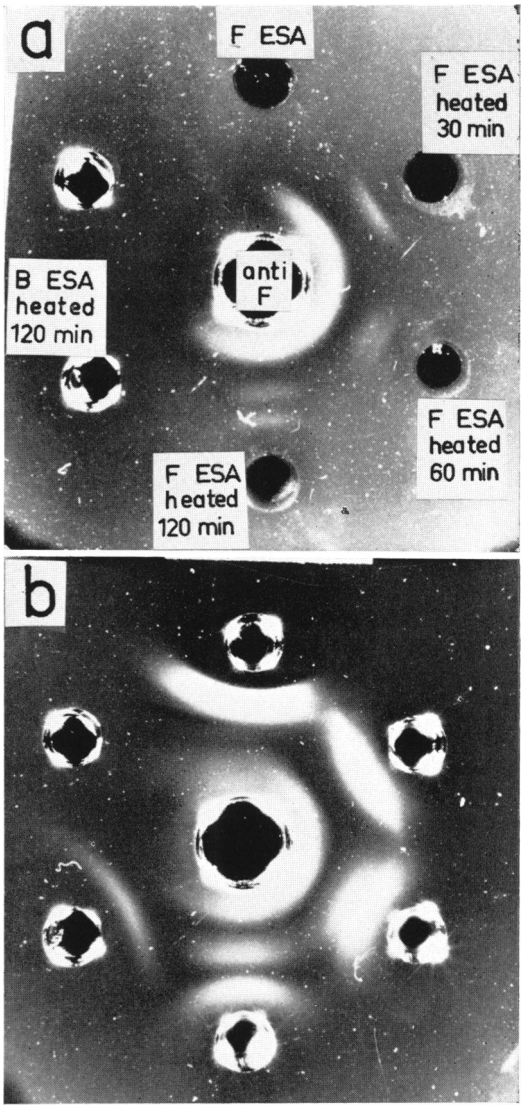


FIG. 7. *Effect of heat (96 C) for 30, 60, and 120 min on a solution (25 mg/ml) in isotonic phosphate buffer (pH 7.3) of the extracellular soluble antigens (ESA) of strain F. Agar (0.75%) in barbital acetate buffer (pH 8.5, ionic strength 0.05). Photographed after (a) 17 hr and (b) 5 days at 25 C. Very similar results were obtained with strain B.*

Fleury and Lange (Dyer, 1956). Oxidation appeared complete by the ninth day, by which time the B antigens had consumed 3.2 mmoles of periodate and the F antigens, 4.4 mmoles. The reactions were stopped by adding 2 ml of ethylene glycol to each flask to destroy the excess perio-

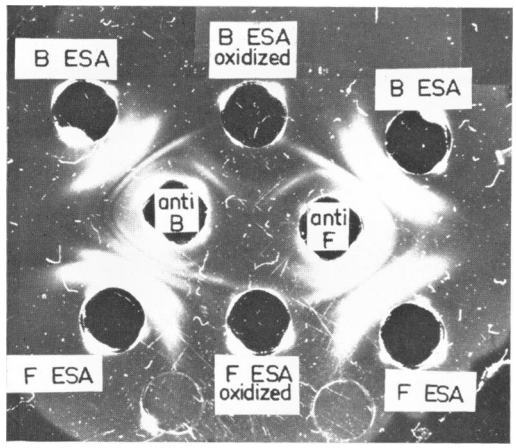


FIG. 8. *Effect of periodate oxidation (9 days with 0.5 M meta-periodic acid at 2 C) on the extracellular soluble antigens (ESA) of strain B and strain F. Photographed after 5 days at 25 C.*

date. After 30 min at room temperature, the solutions were dialyzed for 24 hr against repeated changes of distilled water, and then were dried from the frozen state. The yields of oxidized antigens were 46 and 70 mg from the samples of B and F antigens, respectively.

The oxidized antigens were dissolved in saline to give solutions containing 25 mg/ml, which were tested by immune diffusion (Fig. 8). The oxidized antigens were found to have lost the heat-stable antigens responsible for the dense bands close to the reagent wells. The oxidized antigens of strain B retained some, at least, of the heat-labile antigens that formed the intermediate group of bands, two of which appeared against the homologous antiserum and one in the reaction against the anti-F antiserum. The oxidized antigens of strain F produced only one band of the intermediate group in diffusion against anti-F antiserum.

The stability to heat and susceptibility to periodate oxidation of the antigens that form bands nearest to the antigen and antiserum wells suggested strongly that they are polysaccharides.

This was confirmed by the immune diffusion behavior of the extracellular soluble antigens of the two strains after they had been treated for the removal of protein by the Sevag method. This treatment did not remove all the protein (Table 1), but the residual protein was expected to be denatured and no longer antigenic. The immune diffusion patterns (Fig. 9) obtained with these deproteinized antigens showed the absence of the heat-labile antigens and the presence of only the heat-stable periodate-susceptible antigens which could therefore be identified as protein and polysaccharide antigens, respectively. However, the density of the polysaccharide bands nearest the antigen wells was diminished in all the deproteinized preparations, showing that these antigens were partly affected by the Sevag treatment and

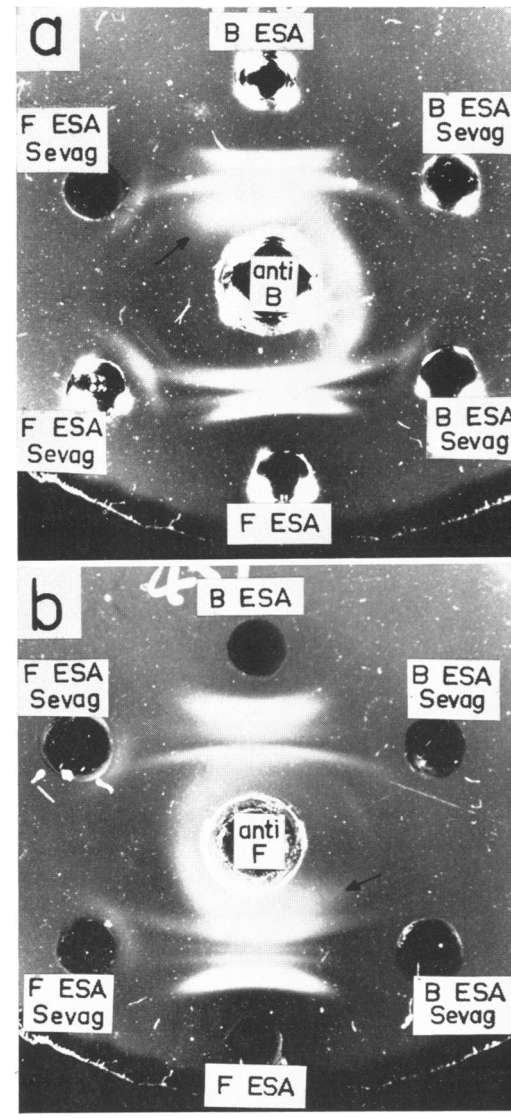


FIG. 9. *Effect of Sevag deproteinization treatment on the extracellular soluble antigens (ESA; 25 mg/ml) of strain B and strain F. Photographed after 2 days at 25 C. Note the strain-specific antigens not affected by the treatment.*

thus may not be polysaccharide alone. The complex nature of these antigens was also shown by the separation into two bands on heat treatment (Fig. 7). A curious feature of these slow-diffusing antigens is their tendency to form only one band when diffused against the homologous antiserum and to form two bands against the heterologous antiserum. This can be seen clearly in the case of the B antigens when diffused against anti-F antiserum (Fig. 6, 8, 9). The phenomenon was seen also, but was less pronounced, in the case of the F antigens diffused against anti-B antiserum.

Strain-specific antigens. The dense crowding and diffuse nature of the precipitin bands in most of the immune diffusion plates (e.g., Fig. 2 and 3) made it difficult to recognize strain-specific antigens by examining the patterns produced by the concentrated solutions of the extracellular soluble antigens. But Fig. 6 and 9 clearly show that the polysaccharide band closest to the antiserum well is specific for each strain of *R. meliloti* without any evidence of cross-reaction with the respective heterologous antisera.

As these specific polysaccharide antigens were detectable in washed cells of the two strains (Fig. 2 and 3), presumably on the cell surfaces despite repeated washing in saline, it is possible to invoke this antigen as the reason for the specificity of the agglutination reaction despite the sharing of so many of the other antigens. This received strong support from the immune diffusion patterns obtained with the absorbed antisera (Fig. 10). The antisera absorbed with homologous cells were depleted of all their antibodies, but the antisera absorbed with heterologous cells contained only the antibodies reacting with the specific polysaccharide antigens.

Influence of cultural conditions on antigenic structure. A matter of obvious importance in studies of the antigenic structure of bacteria is the reproducibility of the immune diffusion patterns. Most of the experience in the present work has been with the extracellular soluble antigens of these two strains of *R. meliloti*. The organisms have been grown many times in the same medium on a rotary shaker, and consistent immune diffusion patterns have been obtained repeatedly.

Changes in cultural conditions have been found, however, to give rise to marked changes in the immune diffusion patterns obtained with these antigens. A number of cultures of strain F were grown in the same medium in a stirred, aerated 10-liter fermentor with different aeration rates to find the conditions for maximal polysaccharide production. Growth under conditions of high aeration gave much poorer yields of cell dry matter and polysaccharide than were obtained with cultures grown with minimal aeration (Dud-

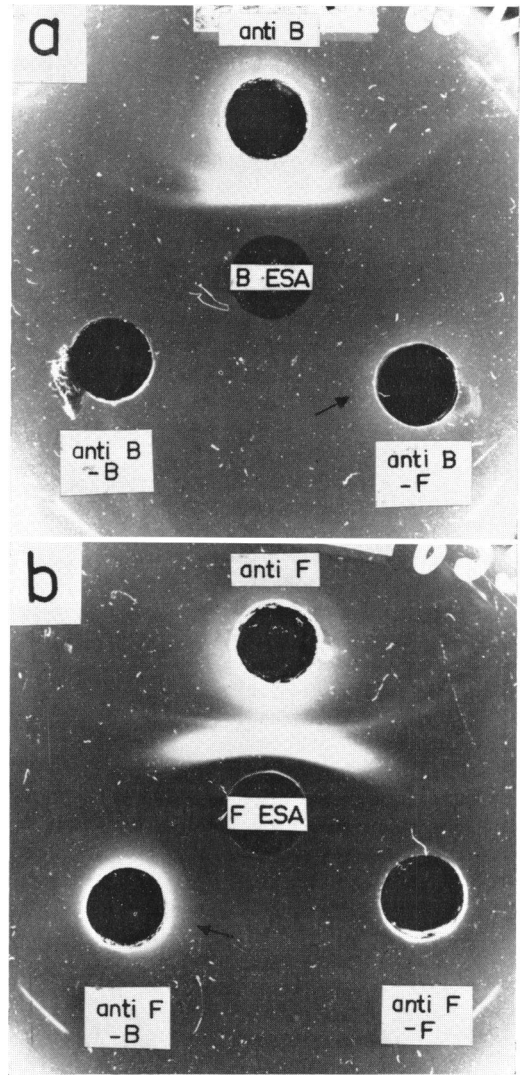


FIG. 10. *Immune diffusion analysis of the extracellular soluble antigens (ESA; 25 mg/ml) of strain B and strain F with absorbed antisera. Anti B — B = anti-B antiserum absorbed with B cells; anti B — F = anti-B antiserum absorbed with F cells; anti F — B = anti-F antiserum absorbed with B cells; and anti F — F = anti-F antiserum absorbed with F cells. Photographed after 3 days at 25 C. Note the specific antigen in each strain detected with the cross-absorbed homologous antiserum.*

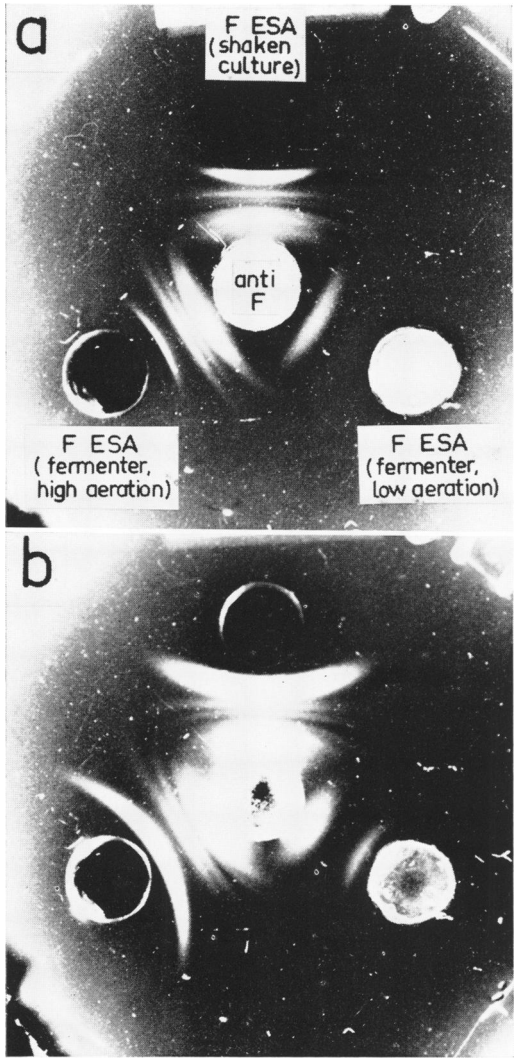


FIG. 11. *Influence of cultural conditions on the extracellular soluble antigens (ESA) of strain F, grown in the same defined medium in (i) 100-ml cultures on a rotary shaker, (ii) 5-liter culture in a fermentor aerated at 10 liters per min, and (iii) 5-liter culture in a fermentor aerated at 0.8 liter per min. The antigens were isolated under identical conditions and tested here at 25 mg/ml. Photographed after (a) 24 hr and (b) 3 days at 25 C.*

man, 1964). The extracellular soluble antigens were isolated from these cultures in the usual way, and immune diffusions were carried out with solutions of identical concentration (25 mg/ml) with the results shown in Fig. 11.

The culture grown under low aeration conditions produced more of the specific polysaccharide than did the well-aerated culture, and thus the 25 mg/ml solution was relatively enriched in the polysaccharide and relatively deficient in the other antigens. There is reason to believe that this difference in antigenic patterns does not arise directly from the difference in aeration conditions but indirectly instead, from the effect of aeration on the pH level of the cultures. Highly aerated cultures became alkaline, whereas those grown under minimal aeration remained neutral (Dudman, 1964).

DISCUSSION

Cross-reactions occur in agglutination systems because bacterial strains, perhaps otherwise completely unrelated, possess similarities in their surface antigens. However, strains that cross-agglutinate do not necessarily share all their other antigens, and it may be postulated that the confidence with which strains may be identified will be increased in proportion to the number of antigens that can be detected. Immune diffusion techniques, with their high resolving power in the analysis of antigens, should offer many advantages over agglutination tests.

Because it is easier to isolate cells from cultures by centrifugation, and to make dense suspensions for use in immune diffusion, it is likely that strain recognition techniques in *Rhizodium* will develop around the use of cells, either intact or disrupted, rather than the extracellular soluble antigens, which require more involved and time-consuming operations such as dialysis and freeze-drying. However, for the purpose of learning something of the nature of the antigens, about which little was hitherto known, it was thought desirable to begin this study of the antigenic structure of *Rhizobium* organisms by examining the extracellular soluble antigens. These could be isolated in relatively large quantities and were expected to be less complex than the cellular antigens.

The extracellular soluble antigens contained multiple components, the enumeration of which is uncertain at the present time because of uncertainty about the number of antigens in some bands. In addition to heterogeneity of the type shown in the splitting of the bands of the slow-diffusing antigens, the hazy flocculent nature of the bands close to the antiserum wells makes detection of separate antigens difficult. Furthermore, the results obtained by immune electro-

phoresis show that the antigens are heterogeneous with respect to electrophoretic mobility.

With the evidence available at present, it may be stated that the extracellular soluble antigens of these two strains of *R. moliloti* contain three groups of antigens on the basis of their location in the immune diffusion plates. Of these, the fast- and slow-diffusing groups have the behavior of polysaccharides, and the intermediate group, that of proteins. The destruction of some of the heat-labile antigens by periodate is not regarded as significant, because the prolonged treatment with concentrated periodic acid would be expected to denature proteins. Although described as polysaccharides and proteins, the antigens may be polysaccharide-protein complexes in which the determinant groups belong to the polysaccharides or proteins, respectively.

The protein antigens and the slow-diffusing polysaccharide antigens are shared by both strains, but the fast-diffusing polysaccharide is not. The strain specificity of this polysaccharide is believed to account for the low degree of cross-agglutination between washed cells of these strains. Despite repeated washing of the cells, the specific polysaccharide was still the predominant surface antigen. This tenacity is surprising in the absence of a microscopically detectable capsule.

The nature of the slow-diffusing antigens is unclear. The location, appearance, and intense whiteness of this group of antigens suggests that they may be the endotoxin or somatic O antigens of these cells. As such, they would be expected to be lipopolysaccharide-protein complexes (Westphal, 1959). The partial removal of these bands by the Sevag treatment suggests the presence of protein as well as polysaccharide. More significantly, the degradative changes caused by heat treatment (Fig. 7) are similar to the type of changes resulting from degradation, by other treatments, of the endotoxins of strains of *Escherichia coli* (Rüde and Goebel, 1962), *Salmonella typhosa* (Rudbach and Johnson, 1962), and *S. enteritidis* (Ribi et al., 1962).

The cause of the "band-splitting" seen when these slow-diffusing antigens react with the heterologous antisera is obscure at present, but it appears to be a manifestation of a phenomenon found also in other bacteria. In a recent study of the immune diffusion patterns of the extracellular antigens of pathogenic clostridia, Ellner and Green (1963) drew attention to two phenomena important in serological studies of this type, namely the occurrence of nonreciprocal reactions and the occasional observation that heterologous reactions may give rise to a greater number of bands than homologous reactions.

The chemical analysis of the unfractionated extracellular reactions is not yet complete. The unpublished evidence available at present suggests that there are no significant differences in the sugar components present in the hydrolysates of the antigens of the two strains. Some similarity in composition would be expected, because all but one of the antigens are common to the two strains, but the specific polysaccharide of each is antigenically distinct and therefore some differences would have also been expected. It is possible, of course, that there may be only subtle differences between the specific polysaccharides, such as in the sequence of the sugars in the molecules, or in the nature of the linkages between them. However, until the individual antigens are isolated and analyzed separately, the significance of the results obtained by analyzing complex mixtures such as these extracellular soluble antigens cannot be assessed.

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